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Full Length Research Paper

Effects of Mn^{2+} levels on the resistance properties of *Bacillus cereus* spores

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In some *Bacillus* species, manganese levels influence the resistance properties of spores. To determine if this was true for *Bacillus cereus*, bacteria were sporulated with different $MnCl_2$ concentrations resulting in spores with 30-fold differences in core Mn^{2+} levels. Spores with different Mn^{2+} levels displayed no differences in resistance to dry heat, UV radiation, γ-radiation, or hydrogen peroxide. However, spores with the lowest Mn^{2+} level were less resistant to wet heat. Overall, Mn^{2+} levels were not a major factor in *B. cereus* spore resistance, and this suggests that this will also be true for the closely related *B. anthracis* spores.

Key words: Manganese (Mn^{2+}), spores, spore resistance, γ-radiation, *Bacillus*, *Deinococcus.*

INTRODUCTION

Spores of *Bacillus* species are extremely resistant to a variety of stress factors including wet or dry heat, UV or γ-radiation, or toxic chemicals, including oxidizing agents such as hydrogen peroxide (H_2O_2) (Setlow and Johnson, 2012). Spore resistance to these agents is due to a number of factors including the proteinaceous spore coats, the low hydration level of the spore core, the high levels of pyridine-2,6-dicarboxylic acid (dipicolinic acid (DPA)) in the spore core, the saturation of the spore genome with the α/β-type small, acid-soluble proteins (SASP), and DNA repair during spore outgrowth. A number of reports indicate that some agents, in particular UVA radiation and γ-radiation, kill growing cells of many aerobic organisms by generating reactive oxygen species (ROS) that damage macromolecules, including proteins and nucleic acids (Avery, 2011; Daly, 2012). Interestingly, elevated Mn levels are associated with increased resistance of growing cells of many bacteria and archea to UVA and γ-radiation, perhaps due to non-enzymatic detoxification of ROS by Mn^{2+} containing small molecules (McEwan, 2009; Daly, 2012).

Previous work has also shown that spore Mn levels have significant effects on the resistance properties of *Bacillus megaterium* spores, with elevated Mn levels associated with increased spore resistance to wet or dry heat, UVC radiation, and hydrogen peroxide, although, Mn levels had no effect on spore γ-radiation resistance (Donnellan and Stafford, 1968; Aoki and Slepecky, 1974; Ghosh et al., 2011). Elevated Mn levels are also associated with elevated resistance of *Bacillus fastidiosus* spores to wet heat (Aoki and Slepecky, 1973). In contrast, *Bacillus subtilis* spores with over a 200-fold range of protoplast Mn levels exhibited no significant differences in resistance to wet or dry heat, γ-radiation or hydrogen peroxide, although, spores with low Mn levels were less resistant to UVC radiation than high Mn spores (Ghosh et al., 2011; Granger et al., 2011). Given the increasing use of γ-radiation as a means for sterilization of foodstuffs and the importance of *Bacillus cereus* as a food-borne pathogen (Setlow and Johnson, 2012), it

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Table 1. Levels of Mn^{2+} in spores prepared in media with different amounts of added $MnCl_2$*.

[$MnCl_2$] Added to sporulation medium (µmol l^{-1})	Mn^{2+} content of spores (µg gm^{-1} dry weight)
0.5	73
1	485
10	1709
100	2311

B. cereus was sporulated at 30°C in Ellar's modified liquid sporulation medium with different $MnCl_2$ concentrations added (Stewart et al., 1981), and the spores were then purified and treated with EDTA (Ghosh et al., 2011; Granger et al., 2011). Spore Mn^{2+} and DPA levels (data not shown) were determined as described (Yi and Setlow, 2010; Ghosh et al., 2011; Granger et al., 2011).

seemed worthwhile to examine the effects of *B. cereus* spore Mn levels on these spores' resistance properties. In addition, given the close relatedness between *B. cereus* and *Bacillus anthracis* (Priest et al., 2004), results with *B. cereus* spores will also likely be applicable to *B. anthracis* spores.

RESULTS AND DISCUSSION

Preparation of *B. cereus* spores with varying levels of Mn

B. cereus was able to form spores in the presence of 0.5 to 100 µmol L^{-1} added $MnCl_2$, resulting in an ~30-fold increase in spore Mn levels in EDTA-washed spores at the highest $MnCl_2$ levels employed (Table 1). As found with spores of *B. megaterium* and *B. subtilis* (Ghosh et al., 2011; Granger et al., 2011), EDTA treatment removed < 2% of Mn from spores prepared with $\leq$ 10 µmol L^{-1} Mn, and only ~ 10% from spores prepared with 100 µmol L^{-1} Mn. These results suggest that ≥ 90% of Mn incorporated into spores prepared with $\leq$ 100 µM Mn is in the spore core, as EDTA should remove all Mn, except for that present in the spore core where most divalent cations are chelated with DPA. Note that even the maximal level of Mn found in spores will chelate < 5% of spore DPA, and DPA levels in spores made with various Mn levels were all within 7% of each other.

Resistance properties of spores with different Mn levels

Analysis of the resistance of spores containing different Mn levels, with the exception of γ-radiation resistance, was carried out at least three times, each time using at least duplicate measurements of spore viability, and always with essentially identical results. Spore treatment with γ-radiation was carried out only once, but the irradiated samples were analyzed at least three times, each using at least duplicate measurements of spore viability, again with essentially identical results. One representative measurement of the resistance of spores prepared with different Mn levels to various agents is shown in Figure 1. In these measurements, values for spore viability are $\leq \pm$ 20%. These results indicate that over a 30-fold range, Mn levels play no notable role in *B. cereus* spore resistance to dry heat, UVC radiation, γ-radiation or H_2O_2 (Figure 1a to d). However, spores with the lowest Mn levels did exhibit lower resistance to wet heat (Figure 1e).

Compared to other *Bacillus* species where the role of Mn on multiple resistance properties has been well-studied, *B. cereus* is more similar to *B. subtilis,* in that, only a single resistance property is affected by Mn (wet heat and UVC radiation, respectively). Why might these species differ in regard to the effects of Mn compared to *B. megaterium*, where multiple resistance properties are sensitive to Mn levels? One potential explanation is that the resistance properties of the different species are related to the number of chromosomes in the spores, since *B. subtilis* and *B. cereus* spores are monogenomic, while *B. megaterium* spores are digenomic (Hauser and Karamata, 1992). Multiple chromosomes in spores would allow for recombinational repair of DNA damage early in spore outgrowth, and it is possible that a component of this process is particularly sensitive to ROS. However, this scenario seems unlikely, as a number of reports indicate that wet heat and H_2O_2 kill spores of *Bacillus* species by damage to proteins, and not by DNA damage (Palop et al., 1998; Coleman et al., 2007, 2010; Setlow and Johnson, 2012). Moreover, even in the case of γ-irradiation there is uncertainty in regard to the type, level and possible importance of DNA damage in spore killing. The level of γ-irradiation-induced DNA damage would be expected to be significantly less in *Bacillus* spores compared to vegetative cells, since the core water content of spores is extremely low (Setlow and Johnson, 2012). Indeed, because of spores' low core water content, spore DNA is predicted to be significantly more resistant to double strand break (DSB) formation by γ-radiation, and Mn-complexes that act as antioxidants may give only minimal protection against γ-radiation (Gaidamakova et al., 2012). As a result, DNA may not even be the lethal target for ROS generated by oxidative stress caused by γ-radiation and other agents (Daly,

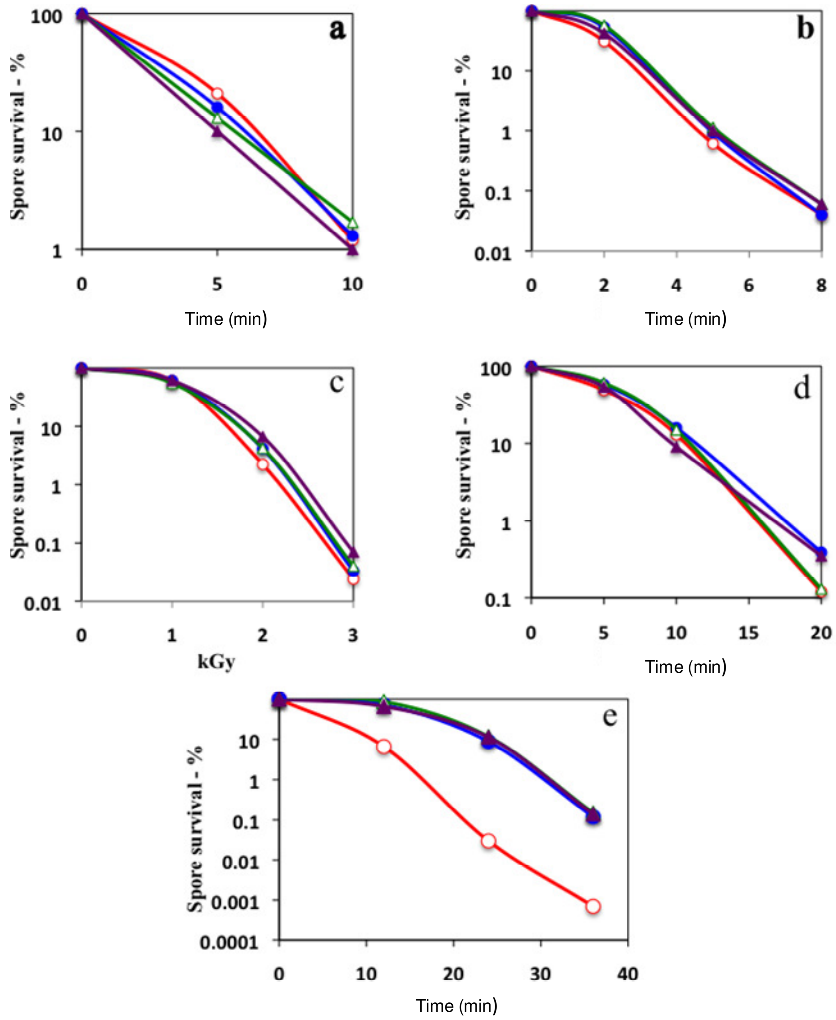

Figure 1. Resistance properties of *B. cereus* spores with different Mn contents. Plots illustrating survival of *B. cereus* spores prepared with varying $MnCl_2$ concentrations following treatment with (a) dry heat at 120°C, (b) UVC radiation at $5x10^{-4}$ J min^{-1} cm^{-2}, (c) varying doses of γ-radiation measured in kiloGrays (kGy), (d) H_2O_2 (5% in 25 mM KPO_4 buffer (pH 7.4) at 23°C) and, (e) wet heat at 87°C are shown. All values were ≤ +/- 20%, and all resistance measurements were made at least twice, with at least duplicate determinations at each time point. The symbols denoting the $MnCl_2$ added to the sporulation medium are: (○) 0.5 µmol l^{-1}; (●) 1 µmol l^{-1}; (△) 10 µmol l^{-1}; and (▲) 100 µmol l^{-1}. Spores of *B. cereus* T (originally obtained from H.O. Halvorson) were prepared at 37°C in liquid medium with different levels of added $MnCl_2$, and harvested and purified as described (Stewart et al., 1981; Ghosh and Setlow, 2010). These spores were free (> 98%) of growing or sporulating cells, germinated spores and cell debris as determined by phase contrast microscopy. Prior to analyses of spore resistance, spore preparations were incubated for 1 h with 10 mM EDTA at 4°C, and then washed thoroughly with water and stored in water at 4°C protected from light. Measurements of spore killing by wet heat, dry heat, UVC radiation, γ-radiation in liquid, or hydrogen peroxide were all carried out as described (Ghosh et al., 2011; Granger et al., 2011).

2012).

Given the above facts, it is not clear why changes in Mn levels would have such different effects on the resistance of spores of *B. cereus*, *B. megaterium* and *B. subtilis* to wet or dry heat, UVC radiation or hydrogen peroxide. The differences in these effects is most striking in examining wet heat resistance, as a low Mn level has no effect on *B. subtilis* spore wet heat resistance, decreases *B. cereus* spore wet heat resistance slightly, and decreases *B. megaterium* wet heat resistance

markedly (Figure 1e; Ghosh et al., 2011; Granger et al., 2011). Why might Mn levels alter or not alter spores' wet heat resistance? While there is no definitive answer for this question, we suggest the following, and highly speculative, scenario. Since wet heat killing of spores of *Bacillus* species is most likely by protein damage (Coleman et al., 2007, 2010), perhaps in *B. megaterium* spores increasing Mn levels protect some key protein whose inactivation by wet heat results in spore death. In *B. subtilis* spores this same protein might be more resistant to wet heat, such that it is wet heat damage to another protein, and one whose stability is insensitive to Mn levels that results in spore death. In *B. cereus* spores, perhaps cumulative wet heat damage to several proteins causes spore death, and Mn levels affect the stability of only one of these proteins.

Two other points are also worth noting on the effects of Mn levels on spore resistance. First, given the close relatedness between *B. cereus* and *B. anthracis* (Priest et al., 2004), it seems likely that Mn levels will also have minimal effects on the resistance of *B. anthracis* spores. Given the interest in the killing and resistance of *B. anthracis* spores, this is a conclusion with significant applied importance. Second, usual media for spore preparation are invariably supplemented with $MnCl_2$, and with concentrations ≥ 10 µmol l^{-1}. At least with spores of *B. cereus* and *B. subtilis*, this Mn concentration results in spores with maximal resistance to all agents examined. Consequently, one does not have to worry that sporulation in media with extremely high Mn levels will give spores of these species with abnormally elevated resistance.

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